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## Postaggregative Differentiation Induction by Cyclic AMP in *Dictyostelium*: Intracellular Transduction Pathway and Requirement for Additional Stimuli

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Cyclic AMP induces postaggregative differentiation in aggregation competent cells of *Dictyostelium* by interacting with cell surface cAMP receptors. We investigated the transduction pathway of this response and additional requirements for the induction of postaggregative differentiation. Optimal induction of postaggregative gene expression requires that vegetative cells are first exposed to 2-4 hr of nanomolar cAMP pulses, and subsequently for 4-6 hr to steady-state cAMP concentrations in the micromolar range. Cyclic AMP pulses, which are endogenously produced before and during aggregation, induce full responsiveness to cAMP as a morphogen. The transduction pathway from the cell surface cAMP receptor to postaggregative gene expression may involve  $\text{Ca}^{2+}$  ions as intracellular messengers. A cAMP-induced increase in intracellular cAMP or cGMP levels is not involved in the transduction pathway. © 1986 Academic Press, Inc.

### INTRODUCTION

cAMP functions in many organisms as an intracellular second messenger for a large number of extracellular stimuli. In the cellular slime mold *Dictyostelium discoideum*, cAMP functions as an extracellular first messenger and acts both as a chemoattractant and as a morphogen. cAMP oscillations control the aggregation process (see Devreotes, 1983) and are very likely also responsible for the control of postaggregative cell movements which lead to slug formation and culmination (Schaap *et al.*, 1984; Kesbeke *et al.*, 1986). Intracellular responses to extracellular cAMP signals have been extensively investigated in the aggregation stage. cAMP is detected by cell surface cAMP receptors (Malchow and Gerisch, 1974). Binding of cAMP to its receptor induces a short transient rise in intracellular cGMP levels, a response which very likely leads to chemotactic movement (Mato *et al.*, 1977; Wurster *et al.*, 1977; Ross and Newell, 1981; Van Haastert *et al.*, 1982). cAMP also induces intracellular synthesis and secretion of cAMP (Shaffer, 1975; Roos *et al.*, 1975). This response ensures transmission of the chemotactic signal through the cell population. Both responses require cAMP concentrations in the nanomolar range and share as a second element an adaptation process, which terminates activation of guanylate cyclase activity after a few seconds and of adenylate cyclase activity after a few minutes, if the stimulus remains present at the same level. The cells deadapt when the stimulus is removed (Dinauer *et al.*, 1980; Van Haastert and Van der Heijden, 1983).

Millimolar cAMP concentrations induce the synthesis of gene products, which are specific for the late aggregation stage (Town and Gross, 1978; Landfear and Lodish, 1980; Mehdy *et al.*, 1983; Chisholm *et al.*, 1984). Postaggregative gene expression comprises a major fraction of gene products specific for spore cells (Barklis and Lodish, 1983; Morrissey *et al.*, 1984; Borth and Ratner, 1983). The induction of postaggregative gene expression by cAMP is also mediated by cell surface cAMP receptors (Schaap and Van Driel, 1985; Van Lookeren Campagne *et al.*, 1986), but in contrast to preaggregative cAMP-induced responses, this response is not affected by adaptation to constant cAMP stimuli. Postaggregative gene expression can be induced as effectively by cAMP pulses as by similar amounts of cAMP provided as a continuous flux or as a single dose. Optimal induction of postaggregative gene products requires stimulation of the cells with cAMP during several hours. An initial cAMP stimulus in the millimolar range is necessary because cAMP is extensively hydrolyzed by extracellular cAMP-phosphodiesterases. It was indicated that steady state concentrations in the micromolar range suffice for induction of postaggregative gene products (Schaap and Van Driel, 1985).

In this study, we investigate whether additional stimuli besides cAMP are required for postaggregative gene expression and we take initial steps to analyze the intracellular transduction pathway which leads to gene expression. It is demonstrated that vegetative cells require a period of exposure to nanomolar cAMP pulses

to obtain full competence for postaggregative differentiation induction. We present some evidence that  $\text{Ca}^{2+}$  ions may function as intracellular messengers of cAMP-induced gene expression, while a cAMP-induced increase of intracellular cAMP and cGMP levels is most likely not involved in the transduction pathway to postaggregative gene expression.

#### MATERIALS AND METHODS

**Materials.** Glycogen, verapamil, 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate-HCl (TMB-8), dansylcadaverine, and 2'-deoxy-cAMP were obtained from Sigma (St. Louis, Mo.). cAMP, glucose-6-phosphate dehydrogenase and phosphoglucomutase were obtained from Boehringer (Mannheim, Federal Republic of Germany). cGMP-RIA kits, [2,8- $^3\text{H}$ ]cAMP, L-[1- $^{14}\text{C}$ ]ornithine, and [ $\alpha$ - $^{32}\text{P}$ ]dCTP were from Amersham (United Kingdom). MUD-1 prespore specific monoclonal antibody was a gift of Dr. M. Krefft and D19 cDNA was kindly supplied by Dr. S. Cohen.

**Organisms and culture conditions.** *D. discoideum*, strain NC4 was routinely used. Mutant N7 of NC4 (Frantz, 1980) was supplied by Dr. P. N. Devreotes and *D. discoideum* XP55 and mutant strain NP368 (Ross and Newell, 1981) by Dr. P. C. Newell. All strains and mutants, were cultured in association with *Escherichia coli* 281 on glucose peptone agar (Schaap and Spek, 1984). Vegetative cells were freed from bacteria by repeated washing with 10 mM Na/K phosphate buffer, pH 6.5 (PB), distributed on nonnutrient agar ( $2 \times 10^6$  cells/cm $^2$ ), and incubated for 16 hr at 6°C. This method yielded a synchronous population of aggregation competent cells which started to aggregate within 1 hr after being transferred to 20°C (Schaap and Van Driel, 1985). Starvation for 16 hr at 6°C is about equivalent to starvation for 6 hr at 20°C.

Cells were collected from the agar, resuspended to  $5 \times 10^6$  cells/ml in PB, and shaken at 150 rpm and 21°C for 1–8 hr. cAMP and various other compounds were added as indicated in the figure legends. At various time intervals, samples of 5 to 10 ml were withdrawn from the suspension; the cells were washed two times with PB, and resuspended to  $10^8$  cells/ml. Aliquots of 100 and 30  $\mu\text{l}$  of this suspension were stored at  $-20^\circ\text{C}$  for the assay of glycogen phosphorylase and prespore antigen, respectively. RNA was extracted from 200- $\mu\text{l}$  aliquots. Ornithine decarboxylase activity was immediately determined in 70- $\mu\text{l}$  aliquots.

**Enzyme assays.** Ornithine decarboxylase activity was determined *in vivo* by measuring the production of  $^{14}\text{CO}_2$  during incubation of  $7 \times 10^6$  intact cells with 1 ml 100  $\mu\text{M}$  L-[1- $^{14}\text{C}$ ]ornithine in PB (Van Lookeren Campagne and Löwik, 1985).

Glycogen phosphorylase activity was measured in 70  $\mu\text{l}$  sonicated cell suspension by means of a linked enzyme

system with phosphoglucomutase, glucose-6-phosphate dehydrogenase, and NADP (Firtel and Bonner, 1972; Schaap and Van Driel, 1985).

The results from both enzyme assays were standardized for the protein content of the cells (Lowry *et al.*, 1951).

**Semiquantitative assay of prespore antigen.** Samples for the assay of prespore antigen were lysed by freeze-thawing and diluted to contain 1  $\mu\text{g}$  protein/100  $\mu\text{l}$ . The amount of a prespore-specific cell surface antigen was measured with a prespore-specific monoclonal antibody (MUD-1) (Gregg *et al.*, 1982), by means of an enzyme-linked immunosorbent assay (Schaap and Van Driel, 1985).

**Semiquantitative assay of prespore specific mRNA.** Cytoplasmic total RNA was isolated from  $2 \times 10^7$  cells by means of phenol extraction (Alton and Lodish, 1977). After ethanol precipitation, the RNA was resuspended in 15% formaldehyde and 0.3 M NaCl in 0.03 M Na-citrate, pH 7.0. The RNA concentration of the extract was determined by measuring the OD $_{260}$  and the extracts were diluted to contain 1  $\mu\text{g}$  RNA/ $\mu\text{l}$ . Aliquots of 10  $\mu\text{l}$  were spotted on nitrocellulose filters using a Minifold II slot blot apparatus (Cheley and Anderson, 1984). D19 cDNA was labeled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP by means of nick translation (Rigby *et al.*, 1977) and the nick-translated probe was hybridized to nitrocellulose filters as described by Thomas (1980). The blots were exposed to X-ray film (RX-NIF Fuji), using intensifying screens. After 24–48 hr of exposure at  $-70^\circ\text{C}$ , the films were scanned with a Joyce Loebel densitometer. Spots derived from hybridization of D19 cDNA to RNA extracted from vegetative cells were used as assay blanks. This method yielded a relative measure of the amount of D19 mRNA in the cell extract.

**cAMP binding assay.** Cells preincubated with various cAMP concentrations were centrifuged for 1 min at 150 rpm and  $0^\circ\text{C}$ ; supernatant fluid was discarded, and remaining fluid was removed from the tubes with tissue paper. The cells were resuspended in 15 ml PB and again centrifuged. This procedure was repeated three times to remove any remaining cAMP. Subsequently, the cells were resuspended to  $10^8$  cells/ml in PB and incubated for 1 min at  $0^\circ\text{C}$  with 10 nM [2,8- $^3\text{H}$ ]cAMP and 5 mM dithiothreitol. After 1 min, the cells were centrifuged through silicone oil and the radioactivity of the pellet was measured (Klein and Juliani, 1977; Schaap and Spek, 1984). Assay blanks, which contained an additional 0.1 mM of cAMP were subtracted.

**Measurement of intracellular cAMP and cGMP levels.** Aggregation competent cells were incubated at  $10^7$  cells/ml in PB in the presence or absence of 0.1 mM 2'-deoxy-cAMP. At various time intervals 0.6 ml of cell suspension was placed on top of 400  $\mu\text{l}$  of silicone oil above 20  $\mu\text{l}$  of

3.5% perchloric acid in 20% sucrose in an Eppendorf tube, which was placed in ice. The tubes were immediately centrifuged for 10 sec at 10,000g; this caused almost instantaneous lysis of the cells as they reached the perchloric acid layer. The tubes were frozen in liquid nitrogen, the tips with the cell pellets in perchloric acid were cut off, and the frozen pellets were placed in fresh tubes. The pellets were neutralized with 20  $\mu$ l of PB and 10  $\mu$ l 50% saturated  $\text{KHCO}_3$  and centrifuged for 2 min at 10,000g. The cAMP and cGMP content of 20  $\mu$ l of supernatant were measured by means of cAMP isotope dilution assay and cGMP-radioimmunoassay, respectively. The data were standardized on the protein content of 0.6 ml cell suspension.

## RESULTS

### Choice of Markers for Postaggregative Gene Expression

Because of their accurate and/or sensitive assay potential, we routinely use three protein markers for postaggregative gene expression: two developmentally regulated enzymes, glycogen phosphorylase (Firtel and Bonner, 1972) and ornithine decarboxylase (Van Lookeren Campagne and Löwik, 1985) and a prespore-specific antigen detected by MUD-1 monoclonal antibody (Gregg *et al.*, 1982; Schaap and Van Driel, 1985). The induction of a postaggregative prespore specific mRNA complementary to a cloned DNA, D19 (Barklis and Lodish, 1983) was measured in some experiments. The appearance of all markers during incubation of aggregation competent cells in shaken suspension, with and without 1 mM cAMP is shown in Fig. 1. Halfmaximal induction of D19 mRNA occurs after about 3 hr of incubation with 1 mM cAMP. Glycogen phosphorylase (GP) is halfmaximally induced after about 4 hr and ornithine decarboxylase (ODC) and prespore specific antigen after about 5 hr. During normal development of aggregation competent cells on solid substratum, D19 mRNA and the three protein markers appear about 1 hr later than in shaken suspension with cAMP. However, the same absolute level of induction is reached after a further 4 hr of development (Schaap and Van Driel, 1985; unpublished results).

The similarity of the effect of cAMP on D19 mRNA induction and on induction of prespore antigen, ornithine decarboxylase, and glycogen phosphorylase validates the use of these proteins as markers for postaggregative gene expression.

### Requirement for a Preceding Exposure to cAMP Oscillations

Aggregation competent cells were found to be very responsive to postaggregative differentiation induction by cAMP. We also examined whether cAMP can induce

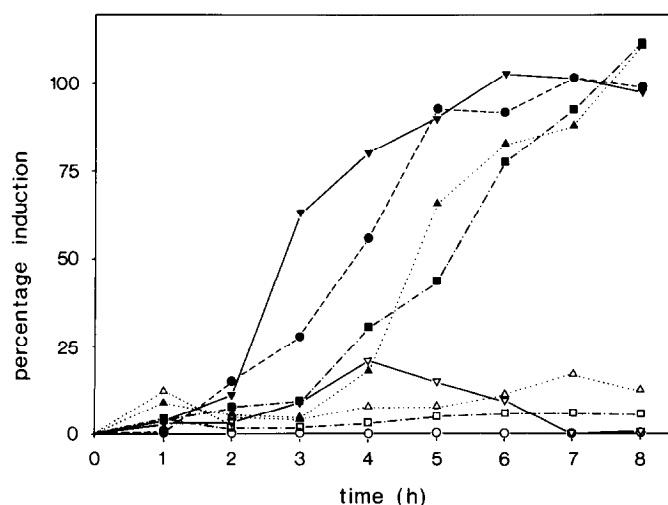


FIG. 1. Induction of several differentiation markers by cAMP. Cells starved for 16 hr at 6°C, were shaken at 150 rpm in PB at  $5 \times 10^6$  cells/ml for 8 hr and 20°C. Open symbols: no cAMP added, closed symbols: 1 mM cAMP added at  $t = 0$  hr. After 4 hr, the cells were collected by centrifugation and resuspended in fresh PB or PB with 1 mM cAMP. Every hour 10-ml samples were withdrawn and prepared for the assay of D19 mRNA content ( $\blacktriangledown$ ,  $\triangledown$ ), glycogen phosphorylase activity ( $\bullet$ ,  $\circ$ ), ornithine decarboxylase activity ( $\blacktriangle$ ,  $\triangle$ ), and prespore antigen ( $\blacksquare$ ,  $\square$ ). For each marker, the data are expressed as percentage of the mean of the absolute induction level at  $t = 7$  and  $t = 8$  hr. The means of two experiments are presented.

postaggregative differentiation directly in vegetative cells in shaken suspension. Figure 2 demonstrates that vegetative cells can be induced to form prespore antigen and ODC, but require more than 12 hr of incubation with 1 mM cAMP (instead of 6 hr), than cells which were first starved on agar for 6 hr at 20°C. This indicates that between the vegetative stage and aggregation, the cells become sensitive for induction of postaggregative differentiation by cAMP. We suppose, that autonomous cAMP oscillations which are generated by starving cells and have been shown to accelerate the competence for aggregation (Darmon *et al.*, 1975; Yeh *et al.*, 1978) also accelerate competence for the induction of postaggregative differentiation (further called, differentiation competence), for instance by inducing cell surface cAMP receptors, which are required for transduction of extracellular cAMP to gene expression (Schaap and Van Driel, 1985).

We investigated the effect of 1, 2, or 4 hr of starvation under various conditions, on cAMP binding activity and on differentiation competence. cAMP binding activity was measured directly after 1, 2, or 4 hr of starvation. Differentiation competence represents in this experiment the levels of prespore antigen and ODC activity induced by an additional 6 hr of incubation with 1 mM cAMP. None of the starvation regimes induced by itself ODC activity and prespore antigen.

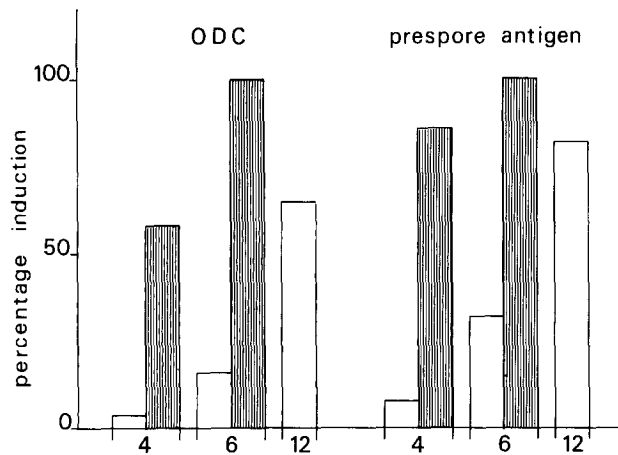


FIG. 2. Differentiation induction in vegetative and aggregation competent cells. Vegetative cells (open bars) or cells starved for 6 hr at 20°C on PB agar (shaded bars) were incubated at  $5 \times 10^6$  cells/ml in PB. Every hour 1 mM cAMP was added to the suspension. After 4, 6, or 12 hr of incubation with cAMP, prespore antigen and ODC activity were measured. The data are expressed as percentage of the induction levels in aggregation competent cells after 6 hr of incubation with cAMP. The means of two experiments are presented.

In *D. discoideum* NC4 about similar differentiation competence is acquired by 4 hr starvation on agar as by 4 hr starvation in shaken suspension in PB (Figs. 3A, B). However, when cells are exposed to cAMP pulses of 0.1  $\mu$ M and 6-min interval during 4 hr starvation in PB, a respectively, three- and twofold increase in subsequent ODC and prespore antigen induction by 1 mM cAMP is obtained. Even 2 hr of exposure to cAMP pulses increases differentiation competence considerably. A continuous influx of similar amounts of cAMP does not accelerate differentiation competence. cAMP binding activity is similarly increased by nanomolar cAMP pulses as differentiation competence (Fig. 3C). Also in this case no increase is observed when cAMP is added as a continuous influx.

Marin and Rothman (1980) described that starvation in a different buffer can mimic the effect of cAMP pulses on acceleration of aggregation competence. In our hands this buffer (MRB) did not induce an increase of differentiation competence compared to starvation in PB (Figs. 3A, B) and neither was an increase in cAMP binding activity observed (Fig. 3C).

Acceleration of differentiation competence and cAMP binding activity by cAMP pulses is shown even more clearly in cAMP relay<sup>-</sup> mutant N7 (Frantz 1980). cAMP-induced activation of adenylate cyclase in this mutant is about 30-fold lower than in parent strain NC4 and is not improved when the cells are exposed to nanomolar cAMP pulses during starvation (Fig. 4B). cAMP-induced activation of guanylate cyclase is about 50% lower than in NC4; this response is completely recovered by treat-

ment with cAMP pulses (Fig. 4A). The mutant does not show oscillatory movement during aggregation and alternately forms loose mounds which disaggregate again.

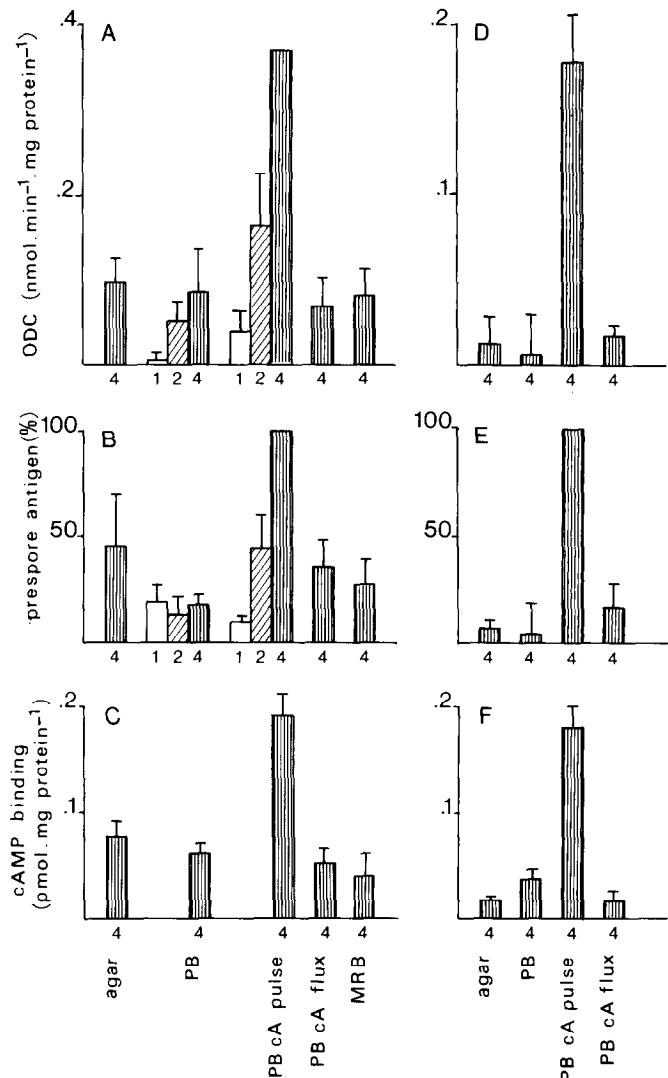


FIG. 3. Induction levels after different starvation regimens. Vegetative NC4 (A-C) and N7 (D-F) cells were incubated on agar at  $10^6$  cells/cm<sup>2</sup> (agar) or shaken at  $5 \times 10^6$  cells/ml and 150 rpm during 1, 2, or 4 hr under the following conditions: (i) in PB (PB), (ii) in PB with 100 nM cAMP added as a short pulse every 6 min (PB, cA pulse), (iii) in PB with a continuous influx of 16 nmole  $\cdot$  l<sup>-1</sup>  $\cdot$  min<sup>-1</sup> cAMP (PB, cA flux), (iv) in a buffer according to Marin and Rothman (1980), which consists of 10 mM NaHPO<sub>4</sub>, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.34 mM CaCl<sub>2</sub>, 0.41 mM MgCl<sub>2</sub>, and 7.5 mM NH<sub>4</sub>Cl at pH 6.8 (MRB). Subsequently, the cells were collected by centrifugation and assayed for [<sup>3</sup>H]cAMP binding activity (C, F) or resuspended at  $5 \times 10^6$  cells/ml in PB and incubated for an additional 6 hr, while 1 mM cAMP was added every hour to the suspension (A, B, D-E). After 6 hr, ODC activity (A, D) and the level of prespore antigen (B, E) were determined. No induction of ODC activity or prespore antigen was observed, when the cells were assayed directly after the initial 1, 2, or 4 hr of pretreatment. Means and SD of two experiments are presented. Numbers below the abscissa indicate the duration of the pretreatment.

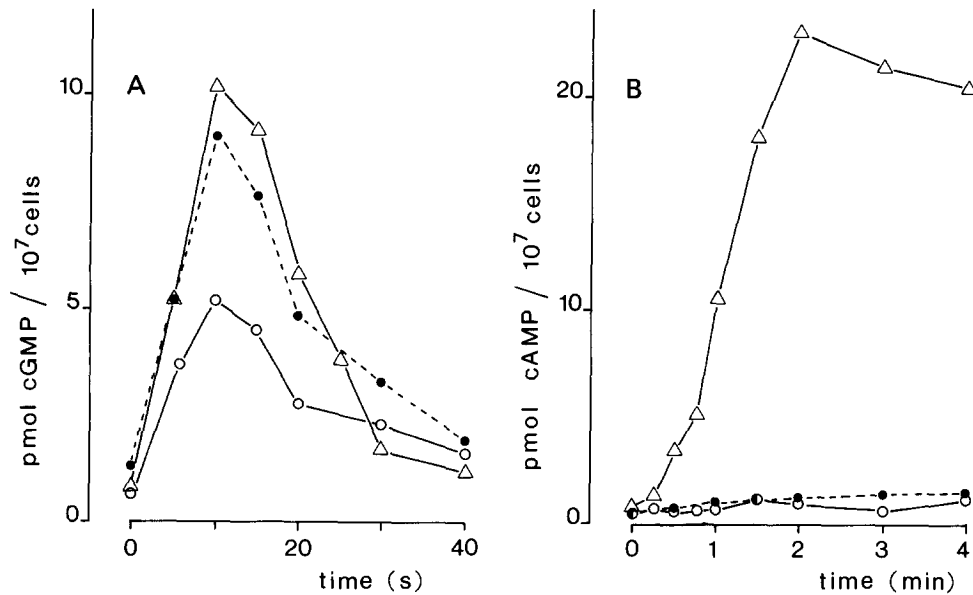


FIG. 4. cAMP relay and the cGMP response in mutant N7 compared to NC-4. (A) cGMP response: 90- $\mu$ l aliquots of  $10^8$  cells/ml were stimulated at  $t = 0$  sec with  $10 \mu$ l  $10^{-6}$  M cAMP. At the indicated time intervals 100  $\mu$ l of 3.5% (v/v) perchloric acid was added. cGMP was measured by means of a commercial cGMP-RIA kit (Van Haastert and Van der Heijden, 1983). (B) cAMP relay response: 90- $\mu$ l aliquots of  $2 \times 10^8$  cells/ml were stimulated at  $t = 0$  min with  $10 \mu$ l  $5 \times 10^{-5}$  M 2'-deoxy-cAMP in 50 mM dithiothreitol. At the indicated time intervals the reaction was terminated with perchloric acid. cAMP was measured by means of a cAMP isotope dilution assay (Van Haastert, 1984). (○) N7 cells and (△) NC-4 cells starved for 6 hr on PB agar at  $2 \times 10^6$  cells/cm<sup>2</sup>; (●) N7 cells shaken in PB at  $10^7$  cells/ml and treated during 6 hr with 100 nM cAMP pulses added at 6-min intervals. The means of two experiments are presented.

However, when N7 cells are treated during 4–6 hr with nanomolar cAMP pulses, followed by a 6-hr treatment with 1 mM cAMP and are subsequently placed on agar, very small slugs and fruiting bodies are formed (Wang and Schaap, 1986). This indicates that besides its relay defect, the mutant is capable of normal development.

Hardly any induction of postaggregative markers can be observed when N7 cells are starved for 4 hr on agar or in suspension and are subsequently treated for 6 hr with 1 mM cAMP. However, when vegetative N7 cells are first treated for 4 hr with 0.1  $\mu$ M cAMP pulses, ODC activity and prespore antigen are similarly induced by cAMP as in wild-type cells (Figs. 3D, E). cAMP binding activity, which remains rather low in N7 during 4 hr of starvation, increases strongly when the cells are pulsed with cAMP during the starvation period (Figs. 3C, F).

Dose-response relationships between 4 hr pretreatment of vegetative cells with cAMP pulses or cAMP flux and the induction of differentiation competence are presented in Fig. 5. In this experiment differentiation competence represents the level of ODC activity induced after an additional 6 hr of incubation with 1 mM cAMP. cAMP pulses accelerate differentiation competence halfmaximally at about 3 nM and maximally at 100 nM. A continuous influx of submicromolar cAMP concentrations inhibits differentiation competence. At higher flux concentrations, the ODC induction level again increases

to the level of cells starved for 4 hr without cAMP. It thus appears that a continuous flux of high cAMP concentrations neither inhibits nor promotes differentiation competence, although it must be realized that in this case vegetative cells were exposed to high cAMP concentrations during 10 hr instead of 6 hr (see also Fig. 2); this prolonged exposure may counteract the inhibition of differentiation competence.

#### *The Intracellular Transduction Pathway: Is Guanylate Cyclase Activation Involved?*

As a first step to analyze the intracellular responses involved in the transduction of extracellular cAMP to gene expression, we investigated whether any known cAMP induced responses are involved. One cAMP induced response is the elevation of intracellular cGMP levels due to transient activation of guanylate cyclase, which is very likely involved in the transduction of extracellular cAMP to chemotaxis (Mato *et al.*, 1977; Wurster *et al.*, 1977; Ross and Newell, 1981; Van Haastert *et al.*, 1982). Possible involvement of guanylate cyclase activation in the transduction pathway to gene expression was studied by using a *D. discoideum* mutant with altered cGMP metabolism (Ross and Newell, 1981). This mutant, NP368, lacks the intracellular cGMP phosphodiesterase. This defect results in the prolonged presence

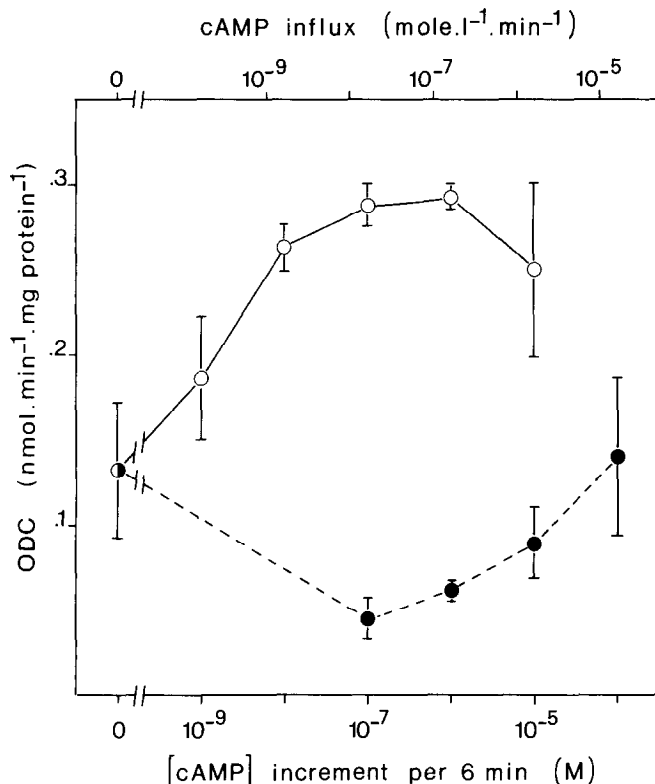


FIG. 5. Dose-response relationships between exposure to cAMP pulses or flux and the acceleration of differentiation competence. Vegetative NC-4 cells were incubated at  $5 \times 10^6$  cells/ml for 4 hr with different cAMP concentrations added as pulses with 6-min interval (○) or as a continuous influx (●). After 4 hr, the cells were washed with PB, re-suspended to  $5 \times 10^6$  cells/ml, and incubated for an additional 6 hr, while 1 mM cAMP was added every hour to the cell suspension. The cells were collected by centrifugation and ODC activity was determined. No induction of ODC activity was observed directly after the initial 4 hr pretreatment with cAMP pulses or cAMP influx. The means and standard deviations of two experiments are presented.

of relatively high intracellular cGMP levels after a cAMP stimulus and is responsible for its phenotype: prolonged chemotactic movement upon stimulation with cAMP. If intracellular cGMP is involved in induction of gene expression, a similar increase in efficiency of cAMP stimuli may be expected. Compared to its parent strain XP55, gene expression in NP368 may require lower cAMP concentrations for a similar induction level.

The dose dependency of the effect of cAMP on induction of glycogen phosphorylase, ornithine decarboxylase and prespore antigen in NP368 and XP55 are presented in Fig. 6. NP368 and XP55 respond to different cAMP concentrations in an almost identical manner. cAMP seems to be somewhat more effective in the parent XP55 than in mutant NP368. It thus seems unlikely that transient cAMP induced changes in intracellular cGMP levels are involved in the transduction of the cAMP signal to gene expression.

### *Is Adenylate Cyclase Activation Involved in Induction of Gene Expression?*

Extracellular cAMP induces a transient activation of adenylate cyclase; intracellular cAMP levels increase, but decrease again within a few minutes because cAMP is secreted (see Devreotes, 1983). This cAMP relay response is involved in the propagation of chemotactic signals. However, as in many higher organisms, the increase of intracellular cAMP levels by activation of adenylate cyclase may function as a second messenger for the effect of extracellular cAMP on gene expression. The activation of adenylate cyclase by cAMP can be inhibited by caffeine (Brenner and Thoms, 1984); halfmaximal inhibition is achieved by 0.1 mM caffeine, while adenylate cyclase activation is completely inhibited at 2 mM. We investigated whether caffeine affects the induction of D19 mRNA, prespore antigen, ODC activity and GP activity by cAMP (Fig. 7). Caffeine was found to cause a minor inhibition at concentrations up to 3 mM. About 50–90% inhibition of induction was observed at 10 mM. This suggests that adenylate cyclase activation is not involved in the induction of postaggregative differentiation markers by cAMP.

Evidence against involvement of adenylate cyclase activation is also derived from the fact that the relay<sup>-</sup> mutant N7 shows normal induction of postaggregative differentiation by cAMP, once the cells have been made competent by a previous exposure to cAMP pulses (Figs. 3A, B). We compared dose-response relationships between cAMP concentration and the induction of GP and ODC activity and prespore antigen levels in mutant N7 and its parent strain NC4 (Fig. 8). No significant differences were evident. The apparently normal induction of postaggregative gene products by cAMP in mutant N7 and the minor effects of the relay inhibitor caffeine indicate that transient cAMP induced adenylate cyclase activation is very likely not involved in the transduction pathway to gene expression.

### *Intracellular cAMP and cGMP Levels*

Another argument against the involvement of the cAMP relay and the cGMP response during induction of postaggregative gene expression is the fact that both these responses adapt when cells are exposed to constant cAMP levels, while the induction of postaggregative gene expression by cAMP does not adapt to a constant stimulus. It is possible that the mechanisms of cAMP induced adenylate cyclase or guanylate cyclase activation contain nonadapting components, which gradually raise the intracellular cAMP or cGMP concentration during prolonged stimulation with cAMP.

To test this hypothesis we incubated aggregation competent cells during 6 hr in the presence or absence

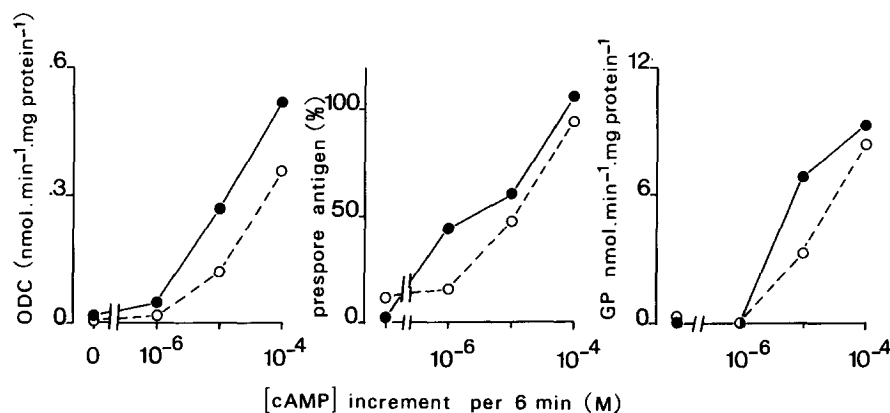


FIG. 6. Differentiation induction in a mutant with altered cGMP metabolism. Wild-type XP55 (●) and mutant NP368 (○) cells, starved for 16 hr at 6°C, were shaken for 5 hr in PB. Every 6 min a short pulse of 1, 10, or 100  $\mu$ M cAMP was added to the cell suspension. After 5 hr, the cells were collected and prepared for the assay of prespore antigen and ODC and GP activity. The means of two experiments are presented.

of 0.1 mM 2'-deoxy-cAMP added with 60-min intervals. 2'-Deoxy-cAMP induces postaggregative gene expression almost as effectively as cAMP (Schaap and Van Driel, 1985), but has a very low affinity for the cAMP binding protein used in the cAMP isotope dilution assay (Van Haastert, 1984). At 2-hr intervals, samples of cells were removed from the incubation mixture and immediately centrifuged through ice-cold silicone-oil into a perchloric acid layer. This was done to separate the cells from the extracellular medium and to prevent any hydrolysis of cAMP or cGMP by intracellular phosphodiesterases. We found that intracellular cAMP levels remained approximately constant during the incubation period (Fig. 9A),

while cGMP levels gradually decreased (Fig. 9B). However, no significant differences were observed in either cAMP or cGMP levels of cells incubated in the presence of 2'-deoxy-cAMP or in the absence of this cAMP derivative. A slow increase in intracellular cAMP or cGMP levels is apparently not involved in the transduction pathway, which leads from extracellular cAMP to postaggregative gene expression.

#### *Involvement of Ca<sup>2+</sup> Mobilization in Differentiation Induction*

In many systems, the interaction of a chemical stimulus with cell surface receptors results in the mobilization of Ca<sup>2+</sup> ions from intracellular or extracellular stores. Ca<sup>2+</sup> ions function as intracellular messengers and their interaction with Ca<sup>2+</sup> binding proteins is considered to be involved in a wide variety of responses (see for a review Rasmussen and Barrett, 1984). We tested whether Ca<sup>2+</sup> mobilization and the interaction of Ca<sup>2+</sup> with Ca<sup>2+</sup> binding proteins are involved in the induction of postaggregative differentiation by cAMP. The effect of the Ca<sup>2+</sup> channel blockers verapamil and TMB-8 (Lee and Tsien, 1983; Chiou and Malagodi, 1975), and the effect of two inhibitors of Ca<sup>2+</sup> binding proteins, trifluoroperazine and dansylcadaverine (Sanchez *et al.*, 1983; Sundan *et al.*, 1983) on induction of glycogen phosphorylase, ornithine decarboxylase, prespore antigen, and D19 mRNA by cAMP were studied.

All drugs were found to inhibit the induction of postaggregative differentiation markers (Fig. 10). Verapamil induced half maximal inhibition at about 50  $\mu$ M and TMB-8 and dansylcadaverine induced halfmaximal inhibition to about 30  $\mu$ M. Trifluoroperazine induced cell death besides inhibition of differentiation induction. Verapamil and TMB-8 reduced cell viability at concen-

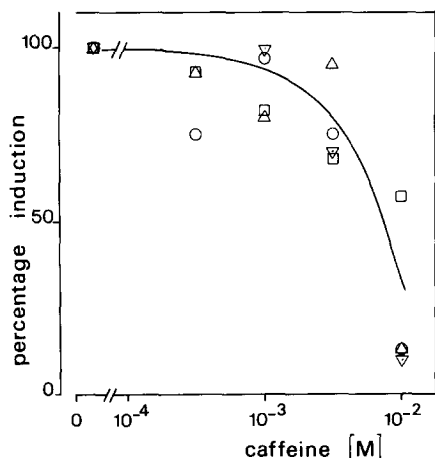


FIG. 7. Effect of caffeine on differentiation induction by cAMP. Cells starved for 16 hr at 6°C, were incubated in PB with different caffeine concentrations at  $5 \times 10^6$  cells/ml for 6 hr. Every hour 1 mM cAMP was added. Every 2 hr the cells were centrifuged for 1 min at 100g and resuspended in a fresh solution of cAMP and caffeine in PB. After 6 hr, the cells were collected and D19 mRNA content (▽), prespore antigen (□), GP activity (○), and ODC activity (△) were assayed. The means of two experiments are presented.



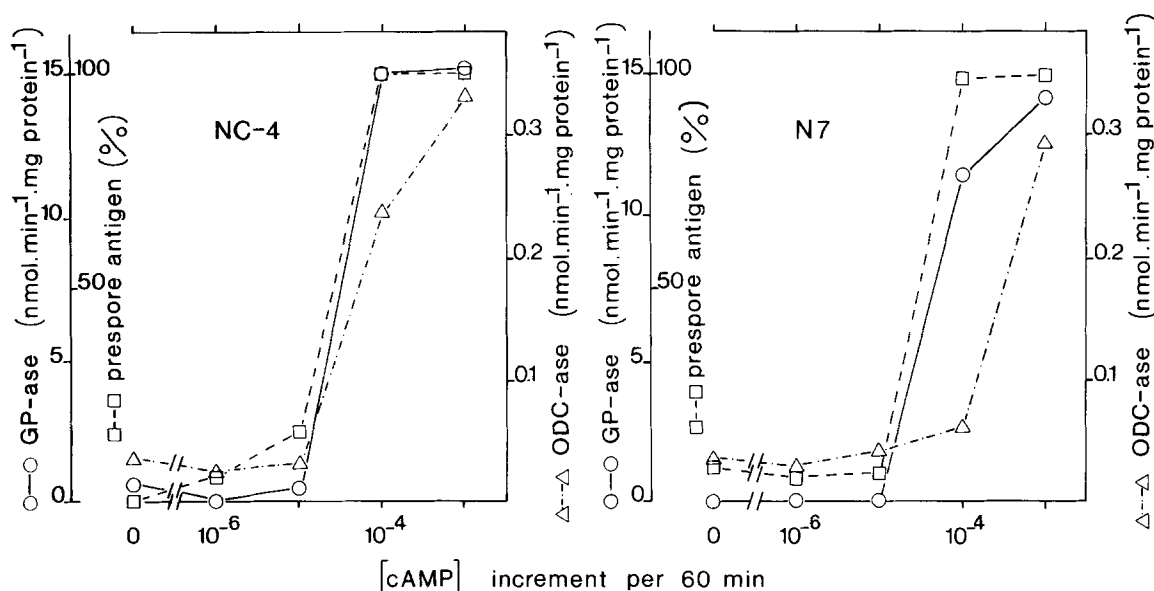


FIG. 8. Differentiation induction in a relay<sup>-</sup> mutant. Wild-type NC4 cells were starved on PB agar for 16 hr at 6°C. Mutant N7 cells were starved at 10<sup>7</sup> cells/ml in PB at 21°C and 150 rpm. During this period a pulse of 0.1  $\mu$ M cAMP was added to the suspension every 6 min. Both NC4 as N7 were further incubated for 5 hr at  $5 \times 10^6$  cells/ml in phosphate buffer and stimulated every 60 min with 1, 10, 100, or 1000  $\mu$ M cAMP. After 5 hr ODC activity ( $\Delta$ ), GP activity ( $\circ$ ), and prespore antigen ( $\square$ ) were assayed. The means of two experiments were presented.

trations of 1 mM and higher. Dansylcadaverine did not affect cell viability at the concentrations used. Aggregation competent cells always form clumps of 50–200 cells when incubated during 6 hr in PB. Neither verapamil, TMB-8, nor dansylcadaverine inhibited the formation of these clumps. When the cells were transferred to nonnutrient agar after 5 hr of treatment with 300  $\mu$ M verapamil, 100  $\mu$ M TMB-8, or 100  $\mu$ M dansylcadaverine, normal aggregates, slugs, and fruiting bodies were formed. Cells exposed to 100  $\mu$ M dansylcadaverine were a few hours delayed in development. The inhibition of postaggregative differentiation induction by verapamil,

TMB-8, and dansylcadaverine indicates that Ca<sup>2+</sup> mobilization and/or Ca<sup>2+</sup> binding proteins may be involved in cAMP dependent induction of postaggregative gene expression.

## DISCUSSION

### cAMP Pulses Induce Competence for Postaggregative Gene Expression

During formation of tight aggregates a large number of new gene products are synthesized, which are for a large part associated with prespore specific differentiation (Alton and Lodish, 1977; Blumberg *et al.*, 1982; Morrissey *et al.*, 1984; Borth and Ratner, 1984; Cardelli *et al.*, 1985). cAMP can induce this type of gene expression in aggregation competent cells in suspension within 4 hr (Fig. 1), but vegetative cells require at least 12 hr of incubation with millimolar cAMP concentrations (Fig. 2). When vegetative cells are treated for 2–4 hr with cAMP pulses in the nanomolar range, the subsequent duration of cAMP treatment, necessary for induction of postaggregative markers is considerably shortened and comparable to induction in aggregation competent cells (Fig. 3). In normal development the cells are exposed to autonomously produced cAMP pulses during aggregation; this signal very likely induces full competence for later differentiation induction. The effect of cAMP pulses was very pronounced in mutant N7, which is impaired

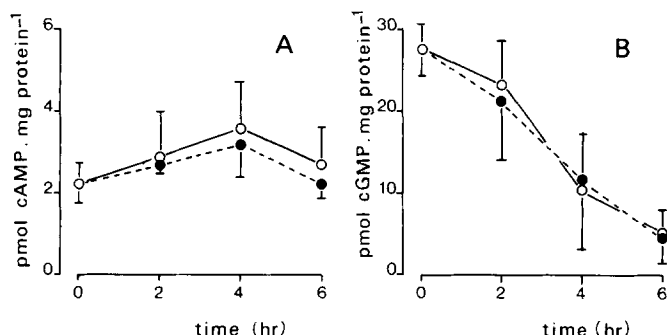


FIG. 9. Intracellular cAMP and cGMP levels. Aggregation competent NC4 cells were incubated at 10<sup>7</sup> cells/ml in the presence (●) and absence (○) of 0.1 mM 2'-deoxy-cAMP. At the indicated time intervals, cell associated cAMP (A) and cGMP levels (B) were determined as described under Materials and Methods. The means and SD of two experiments performed in duplicate are presented.

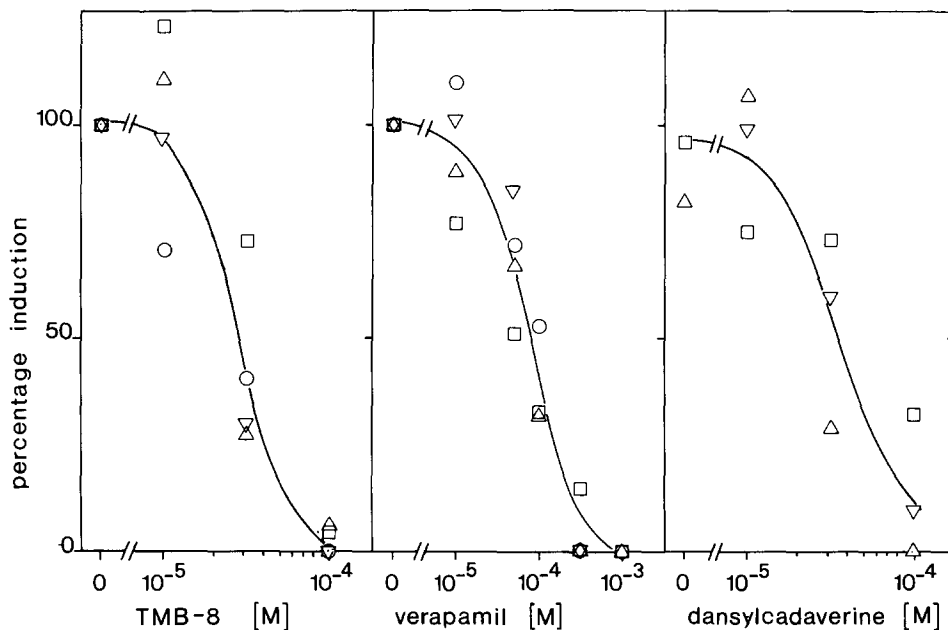


FIG. 10. Effect of dansylcadaverine, TMB-8, and verapamil on differentiation induction. NC4 cells starved for 16 hr at 6°C were incubated at  $5 \times 10^6$  cells/ml in PB. cAMP (1 mM) was added every hour. Various concentrations of TMB-8, verapamil, and dansylcadaverine were added at the onset of incubation. Every 2 hr, the cells were centrifuged and resuspended in a fresh mixture of inhibitors and cAMP. After 6 hr, GP activity (O), ODC activity ( $\Delta$ ), prespore antigen ( $\square$ ), and D19 mRNA ( $\nabla$ ) were measured. The means of two experiments are presented.

in its relay system and therefore does not produce cAMP pulses autonomously.

Competence for postaggregative differentiation appears to be similarly enhanced by cAMP pulses as the appearance of cellular components which are connected with the aggregation process, such as cell surface cAMP receptors, contact sites A and cell surface associated cAMP phosphodiesterase (Darmon *et al.*, 1975; Yeh *et al.*, 1978, Fig. 3). This is very likely a consequence of the fact that the induction of postaggregative gene expression by cAMP requires the presence of cell surface cAMP receptors (Schaap and Van Driel, 1985).

Halfmaximal induction of differentiation competence was achieved with pulses of 3 nM each 6 min, a continuous flux of similar amounts of cAMP per unit time inhibited the induction of differentiation competence. Similar results are reported for induction of cAMP receptors, contact sites A and cell surface associated phosphodiesterase; the inhibitory effects of continuous influx have been attributed to inhibition of autonomous cAMP oscillations (Yeh *et al.*, 1978).

Although nanomolar cAMP pulses accelerate the acquisition of differentiation competence considerably, they are no absolute requirement. When vegetative cells are incubated for very long periods with millimolar cAMP concentrations, they will ultimately synthesize postaggregative gene products (Figs. 2, 5). It is unlikely that millimolar cAMP concentrations also induce differentiation competence; i.e., cAMP receptors, since

constant high cAMP concentrations are known to induce receptor down-regulation (Klein and Juliani, 1977); it is more likely that the small amount of cAMP receptors, which are present on vegetative cells, will transduce a sufficiently strong signal for synthesis of postaggregative gene products when the cells are stimulated for very long periods with cAMP.

Several studies indicate that cell contact formation or other cellular secretion products besides cAMP are required for postaggregative prespore specific gene expression (Blumberg *et al.*, 1982; Okamoto, 1985; Mehdy and Firtel, 1985; Finney *et al.*, 1985). In our experiments aggregation competent cells always form tight cell clumps during incubation in PB, regardless of the presence of cAMP. We can therefore not exclude an additional requirement for short-range intercellular interactions.

#### *Analysis of the Transduction Pathway*

We also investigated the transduction pathway leading from extracellular cAMP to postaggregative gene expression. It was proposed that cAMP induces postaggregative gene expression by leaking into the cell and interacting with intracellular cAMP binding proteins as cAMP-dependent protein kinase (Sampson *et al.*, 1978; Kaleko and Rothman, 1982; Schaller *et al.*, 1984). Recent experiments with the cAMP derivative 8-bromo-cAMP show that this is not the case. This derivative is less

polar than cAMP and may therefore enter the cell more easily (Van Haastert *et al.*, 1983); 8-bromo-cAMP has furthermore a twofold higher affinity than cAMP for intracellular cAMP binding proteins (De Wit *et al.*, 1982). However, 8-bromo-cAMP induces postaggregative gene expression about 100 times less effectively than cAMP (Schaap and Van Driel, 1985). This argues against induction caused by direct leakage of cAMP into the cell.

We investigated whether any of the early cAMP induced responses as transient activation of adenylate cyclase or guanylate cyclase are involved in the transduction pathway leading to postaggregative gene expression. This is most likely not the case. Involvement of guanylate cyclase activation is opposed by the observation that a mutant with strongly altered cGMP metabolism responds similarly to induction of postaggregative differentiation by cAMP as its parent strain (Fig. 6) and by the observation that intracellular cGMP levels do not increase when aggregation competent cells are incubated for 6 hr with 0.1 mM 2'-deoxy-cAMP (Fig. 9A); a treatment which induces optimal synthesis of postaggregative gene products (Schaap and Van Driel, 1985).

Involvement of adenylate cyclase activation is also unlikely; (i) intracellular cAMP levels do not increase significantly while cells are incubated with 0.1 mM 2'-deoxy-cAMP, (ii) caffeine, an inhibitor of cAMP-induced adenylate cyclase activation, does not counteract cAMP-induced postaggregative gene expression at concentrations which completely inhibit adenylate cyclase activation (Brenner and Thoms, 1984; Fig. 7), (iii) relay<sup>-</sup> mutant N7, which is seriously impaired in cAMP-induced adenylate cyclase activation, is not impaired in cAMP-induced synthesis of postaggregative gene products (Fig. 8).

Our finding that cAMP pulses accelerate the appearance of cAMP receptors and the acquisition of postaggregative differentiation competence in NC4 as well as in relay<sup>-</sup> mutant N7, indicates that cAMP induced adenylate cyclase activation is also not involved in the acquisition of differentiation competence, which is induced by cAMP pulses at the postvegetative stage of development. Wurster and Bumann (1981) came to similar conclusions concerning the acquisition of aggregation competence by cAMP pulses.

#### *Ca<sup>2+</sup> Ions May Function as Intracellular Messengers*

We obtained some evidence that Ca<sup>2+</sup> ions may function as intracellular messengers for differentiation induction by cAMP. Differentiation induction by cAMP was rather sensitive to inhibition by the Ca<sup>2+</sup> channel blockers verapamil and TMB-8, and to dansylcadaverine, an inhibitor of Ca<sup>2+</sup>-binding proteins. Dansylcadaverine and TMB-8 inhibited differentiation induction com-

pletely at about 100  $\mu$ M and verapamil at 300  $\mu$ M, which is in the concentration range reported for their inhibitory effects in other organisms (Chiou and Malagodi, 1975; Sundan *et al.*, 1983; Della Bianca *et al.*, 1985). TMB-8 was shown to inhibit activation of guanylate cyclase in *Dictyostelium* (Europe-Finner and Newell, 1984), but only at millimolar concentrations. Millimolar TMB-8 concentrations were found to cause cell death during our long-term incubations. Although the inhibitory effects of TMB-8, verapamil, and dansylcadaverine indicate the involvement of Ca<sup>2+</sup> ions in the transduction pathway, it must be realized that Ca<sup>2+</sup> ions are involved in such a large variety of intercellular processes, that inhibition by these drugs possibly represent pleiotropic effects and not a direct blockage of the specific transduction pathways. It was for instance recently reported that TMB-8 inhibits respiration in *D. discoideum* (Europe-Finner *et al.*, 1985).

#### *Involvement of Adaptation Components of Guanylate and Adenylate Cyclase Activation?*

A remarkable difference distinguishes the induction of postaggregative differentiation by cAMP from earlier cAMP-induced responses such as activation of adenylate cyclase and guanylate cyclase and the acceleration of both aggregation and differentiation competence: The latter responses are only induced by an increase in the ambient cAMP concentration or by alternate increases and decreases. This is due to the fact that these responses adapt to constant or decreasing cAMP concentrations (Dinauer *et al.*, 1980; Van Haastert and Van der Heyden, 1983). However, postaggregative differentiation is induced equally well by continuous as by intermittent stimulation with cAMP (Schaap and Van Driel, 1985) and is therefore not subjected to adaptation. This is another argument against involvement of cAMP induced activation of adenylate cyclase and guanylate cyclase in the induction of postaggregative gene expression. However, although the induction response does not make use of the excitatory components of adenylate cyclase and guanylate cyclase activation, this response shares one property with the adaptation components of both responses: all require continuous occupation of cAMP receptors during prolonged periods (Dinauer *et al.*, 1980; Van Haastert and Van der Heijden, 1983). It is therefore possible that the transduction pathway which leads to gene expression is shared with either one of the adaptation components of the cAMP relay or the cGMP response.

As yet no definite conclusions concerning the transduction of extracellular cAMP signals to gene expression can be drawn. Our results indicate that transient cAMP-induced adenylate and guanylate cyclase activation are

not involved. cAMP does also not induce a slow increase in intracellular cAMP or cGMP levels and involvement of cAMP or cGMP in intracellular signal transduction is therefore very unlikely. A hitherto unknown intracellular response or a response shared with the adaptation components of adenylate or guanylate cyclase and/or involving  $\text{Ca}^{2+}$  ions as intracellular messengers remain as possibilities.

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